AN INVESTIGATION AND ANALYSIS OF PAST, PRESENT, AND FUTURE RESEARCH ON THE DENGUE VIRUS IN BANGKOK, THAILAND EMPHASIZING THE RESEARCH PREVIOUSLY CONDUCTED AT THE ARMED FORCES RESEARCH INSTITUTE OF MEDICAL SCIENCES DURING THE SUMMER OF 2011

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CASSADY MICHELAINA HOSSENLOPP

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ABSTRACT

The dengue virus is the most common arbovirus in tropical and subtropical regions of the world. After analyzing the evolutionary history of the dengue virus, the health risks it presents, prevention methods, and the progress and setbacks that are currently being made in vaccine development, scientists understand the important role detection plays in combatting the virus. The research conducted in this thesis attempts to improve detection of the dengue virus using samples that were previously shown as negative by conventional RT-PCR but positive by ELISA. After designing new primers, conventional RT-PCR was able to detect 22% of the dengue mutants. The results from qRT-PCR, ELISA, and conventional RT-PCR were compared with the clinical data to recognize any possible trends between viral symptoms and the percentage of detection as well as account for possible differences in sensitivity.

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INTRODUCTION

Overview. According to the World Health Organization (WHO), the dengue virus poses a threat to over 2.5 billion people, about 40% of the world's population. The Center for Disease Control and Prevention (CDC) estimates that every year the dengue virus affects over 100 million people, causing undifferentiated febrile illness, dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. These conditions result in approximately 500,000 hospitalizations and 24,000 deaths. Unfortunately, there is no vaccine to protect the human population from the virus, but researchers are working diligently at developing one in order to combat this threat to global humanity (Srikiatkachorn et al., 2010; Poersch et al., 2005).

The dengue virus is the most common arboviral infection of humans in tropical and subtropical regions of the world and is therefore considered the most important human pathogen of the arboviruses (Gibbons, 2010; Weaver and Vasilakis, 2009). The four serotypes of the virus are members of the genus *Flavivirus* and are spherical, lipid-enveloped particles approximately 50 nm and have a genome made up of a single positive-strand RNA (Guzmán and Kourí, 2004; Sadon et al., 2008). The virus is primarily transmitted by female *Aedes aegypti* but can also be transmitted by *Aedes albopictus*, both of which are very small mosquitoes that are known to occupy the tropical and subtropical regions of the world (Sadon et al., 2008).

The dengue virus presents a troubling uniqueness in that there are four closely related serotypes known as DEN-1, DEN-2, DEN-3, and DEN-4. If infected by one serotype, the person gains lifelong immunity to that particular serotype but if infected a second time with

one of the other three serotypes more than 2-3 months after the primary infection, there is a higher risk of developing Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) which are more severe than Dengue Fever (DF) (WHO, 2009). This is due to a phenomenon known as antibody-dependent enhancement (ADE) in which the antibodies produced during the primary infection actually aid in the entry of the second virus into the host cells resulting in a more severe form of the disease (Sadon et al., 2008). This phenomenon causes clinical complications as well as complications in vaccine development.

As the virus continues to evolve and spread to various regions around the world, the methods utilized in research facilities must be efficient in the detection of dengue. This thesis presents previously conducted research to assess and improve the detection of the dengue virus circulating in Thailand using different laboratory tests. In addition to the main study, this thesis also highlights the importance of the study by connecting it to a variety of previously conducted studies and emphasizes its relevance to the common person.

Epidemiology: History and Effects on Health in Thailand. In order to understand the dengue virus, one must investigate its origin in human populations around the world and its dramatic impact on Thailand. In addition, the investigation must include when the virus first caused symptoms in humans and how the virus made its transition to the vulnerable human host.

There is no agreement in the literature as to when the dengue virus first appeared because the symptoms experienced in humans are not distinctly diagnostic (Holmes and Twiddy, 2003). In a previous study, scientists noted that the earliest record of an infection with the dengue virus was reported in 992 A.D. in an ancient Chinese medical encyclopedia.

A more recent article provides evidence that dengue infections date back even further to the 3rd century in China (Holmes and Twiddy, 2003; Weaver and Vasilakis, 2009). These records referred to the disease as "water poison" because it was associated with flying insects that surrounded water and caused fever, rash, arthralgia, myalgia, and sometimes hemorrhaging (Weaver and Vasilakis, 2009). In the late 18th century, a disease resembling dengue caused intermittent epidemics in Asia and the Americas (Holmes and Twiddy, 2003). It is suspected that by the late 19th and early 20th centuries the dengue virus had made its way across most of the tropical and subtropical areas of the world that it currently affects today (Holmes and Twiddy, 2003). However, it was not until a little over 50 years later that the first well documented outbreak of DHF impacted Manila, Philippines. In 1958, an even larger outbreak struck Bangkok, Thailand, the country of focus for this study (Barbazan et al., 2002; Holmes and Twiddy, 2003).

There are approximately 100 million cases of dengue infection occurring globally. The case fatality rate can be as high as 10-15% in certain countries (Gubler, 2002). Since the first DHF outbreak experienced by the city of Bangkok in 1958, Thailand has continually experienced an increase in the number of dengue infections. Children, especially under the age of 15, demonstrate the highest incidence of virus infection (Barbazan et al., 2002; Chareonsook et al., 1999; Wichmann et al., 2011). According to an article published in the *Bangkok Post* on November 5, 2012, Dr. Suwitch Dhammapalo, director of Disease Control Office 12 in Songkhla, stated that since the beginning of 2012 the number of people that had confirmed infections with the dengue virus exceeded 55,000 with 52 fatalities as of October 30, 2012 (Tropical Medical Bureau, 2012). It is very apparent from this data that the dengue virus still poses a grave health threat to Thailand.

Clinical Symptoms Manifestations. Since dengue infections are very common in Thailand and are quite complicated because of the similarities they share with other febrile diseases, Thai physicians and nurses are thoroughly trained to quickly diagnose and treat the signs and symptoms of a suspected dengue patient (Chareonsook et al., 1999; Wichmann et al., 2011). Infections caused by the dengue virus are so common that certain hospitals, such as the Queen Sirikit National Institute of Child Health, have their own dengue ward staffed by physicians that specialize in dengue treatment. This practice increases the efficiency of diagnosing and treating patients suffering from the disease. To diagnose and administer treatment for DF, DHF, and DSS, medical professionals must first recognize the particular symptoms and signs that the virus presents in its host.

Dengue infection may be asymptomatic, but most of the time it causes undifferentiated fevers, DF, DHF, and DSS. Most physicians base their diagnosis on the criteria provided by the WHO using clinical and hematological characteristics (Chareonsook et al., 1999; Sadon et al., 2008). According to the WHO criteria, dengue is suspected when the patient presents a high fever (usually 40°C or 104°F) and at least two of the following signs or symptoms: severe headache, pain behind eyes, muscle and joint pain, nausea, vomiting, swollen glands, or rash. These symptoms can exist anywhere between 2-7 days and usually occur a few days after a mosquito bite.

DHF and DSS are usually more apparent, but can be difficult to assess, and will rapidly escalate and become dangerous. People experiencing these diseases are usually hospitalized and monitored closely. In the cases of DHF, the patient first experiences symptoms similar to DF but within a few days the person's temperature may drop slightly, which gives patients hope that they may be getting better. However, a variety of more serious

symptoms continue to occur. These include enlarged liver, thrombocytopenia (platelet count of <100,000/mm^3), rise in hematocrit >20%, bleeding gums, petechiae, increased rate of breathing, fatigue, and hypotension (Bakshi, 2007; Guzmán and Kourí, 2004; Sun et al., 2011; WHO, 2013). At that point, complications arise in the patient and the risk of DSS and death is increases due to respiratory distress, plasma leakage, severe hemorrhaging, and circulatory failure (Bakshi, 2007; WHO, 2013). Patients usually recover as long as they receive sufficient intravenous fluid and electrolytes, but death can occur if this process is inadequate or delayed (Guzmán and Kourí, 2004). A major protocol when treating patients with suspected DF or DHF is to avoid aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs if administered to a patient would only increase the severity of their symptoms as they can increase the risk of internal bleeding due to their ability to inhibit blood clotting. Acetaminophen is the preferred treatment for pain relief and fever reduction (Bakshi, 2007).

Current Prevention for Dengue. There is currently no licensed vaccine accessible to the public for the dengue virus. Even if a licensed vaccine were released, no sufficient distribution method exists to vaccinate the 2.5 billion people at risk for the virus (Chao et al., 2012). Surveillance and control systems must also be strengthened (Guzmán and Kourí, 2003). By understanding the modes of transmission and the behavior of the dengue virus vector, medical professionals can utilize resources to keep the rate of dengue virus infections from increasing. This will help contain the virus in areas where it is already endemic and prevent it from moving to regions of the world that have yet to experience its effects.

Transmission can begin if a female mosquito ingests the blood of a person who currently has the dengue virus circulating in his or her body. The mosquito can become

infected with the virus itself. If this happens, the dengue virus incubates in the mosquito's body for approximately 8-12 days and then can be transmitted to another human through a female mosquito bite. To make matters worse, the mosquito remains infective for the rest of its life and can pass the virus onto its offspring, making them capable of transmitting the virus to humans as well (Parker and Holman, 2012; WHO, 2009).

Since mosquitoes, particularly *A. aegypti*, are the vectors for the dengue virus, prevention can be achieved (to some extent) by avoiding contact with mosquitoes in areas where the dengue virus is considered endemic. Precautions can include application of repellant containing DEET or Icaridin and wearing long sleeves and pants if planning to be outdoors for an extended period of time especially in the daylight hours, which is primarily when *A. aegypti* feeds (WHO, 2009). Mosquito netting around beds is highly encouraged to help prevent mosquitoes from biting individuals while asleep as well as installing screens on windows and doors to keep mosquitoes from entering buildings. Another important method of preventing the habitation of mosquitoes is to quickly rid property of any stagnant water that has collected after a rainstorm (Jansen and Beebe, 2010). Female mosquitoes lay their eggs on or around water surfaces near human habitation. Once the eggs develop into an embryo they can withstand dry conditions for up to a year and may develop into adults during the next rainy season (Jansen and Beebe, 2010). Lastly, it is important for people who are suspected of having DF or DHF to avoid mosquitoes more than the uninfected individual so that they don't contribute to the mosquito-human-mosquito cycle.

In addition to these methods of protection, organizational and governmental efforts to reduce the risk of mosquito proliferation in dengue endemic regions have been put in place in recent years. Environmental management encourages a street cleansing system which is

responsible for disposing of any waste containers that have the potential to accumulate water and clearing the drains so that they do not become places for larva to develop (WHO, 2009). As *A. aegypti* is an urban mosquito which can utilize buildings within the city as reproduction sites, many areas where dengue is endemic have adopted new regulations for the construction of buildings. In Singapore, for example, roof gutters are no longer permitted on buildings and those already in place must be removed or thoroughly maintained (WHO, 2009). The WHO also recommends that larvicides and insecticides be sprayed around homes and other buildings two to three times a year during the morning or late afternoon. This method of control is fairly effective but can be toxic to humans; therefore, dengue management services must collaborate with epidemiological surveillance systems to ensure that pesticides are applied at the beginning of a dengue outbreak which typically happens during the rainy seasons, usually May-November, depending on the region.

Reducing the spread of dengue by travelers is important in decreasing the global expansion of the virus and preventing outbreaks from occurring in other countries. For example, the deployment of troops during WWII correlated with the rapid globalization of dengue through the transportation of mosquitoes on ships, aircrafts, and automobiles (Wilder-Smith and Gubler, 2008). Today's increased number of travelers from around the world has led to the introduction of mosquito abatement programs in international airports. These programs conduct routine sprayings of insecticides within the airport and passenger cabins of the aircrafts (Wilder-Smith and Gubler, 2008). Despite the installation of dengue prevention programs to help with the transmission and infection rate of the dengue virus, it is impossible to protect the world's population from the virus. With this being the case, the race to license an effective DENV vaccine will continue.

Evolution of the Virus. Historical records and research provide evidence that the dengue virus did not just suddenly emerge as the endemic and epidemic problems that 40% of humans face today. Scientists have observed the gradual development of dengue infections in humans by reconstructing a molecular time-scale demonstrating its evolution. By estimating the nucleotide substitution rates of 120 envelope genes, Holmes and Twiddy (2003) revealed that the dengue virus originated approximately 1,000 years ago as the "water poison" reported in Chinese medical encyclopedias. In the same study, they also discovered that cross-species transmission from monkeys to humans occurred within the last 125 to 320 years (depending on the serotype). This led researchers to suggest that prior to the distinct divergence the virus showed only occasional outbreaks in human populations. It was suggested that the clear "break" into what is today the dengue virus's major host was most likely caused by the rapid increase in human populations, urbanization and traveling. In addition, scientists have found a strong relationship between the period of World War II and the eruption of the dengue virus as a global health concern (Holmes and Twiddy, 2003; Wilder-Smith and Gubler, 2008).

The site of the original cross-species transmission took place has been the subject of much controversy. Previous studies, established that *A. aegypti* have lived in urban areas, but researchers have discovered other mosquito species that have the ability to transmit the dengue virus to non-human primates. This mechanism is referred to as a sylvatic transmission cycle (Holmes and Twiddy, 2003; Wilder-Smith and Gubler, 2008). One area where these transmission cycles have been observed is Asia. In this case, the principal vectors were from the genus *Ochlerotatus* with its main hosts from the *Presbytis* and *Macaca* species (Holmes and Twiddy, 2003). The other area that displayed sylvatic transmission

cycles was in West Africa where various *Aedes* species were able to transmit dengue to *Erythrocebus patas* monkeys (Holmes and Twiddy, 2003).

From the discovery of these sylvatic transmission cycles, the possibility of cross-species transmission began to emerge as an explanation which led to strain investigation in monkeys. These studies revealed that the DENV-2 and DENV-4 strains in Asian and West African monkeys are related to those of human strains, which may also be true of DENV-1 (Holmes and Twiddy, 2003). In Malaysian monkeys, researchers have found antibodies for DENV-3 suggesting there is or once was a DENV-3 sylvatic transmission cycle as well (Holmes and Twiddy, 2003).

Due to these observations a debate arose over Africa versus Asia as the origin of the dengue virus. Some scientists claim the virus emerged in Africa. This is based on the evidence that most other mosquito-borne flaviviruses circulate only in Africa and infect primates. Furthermore, *A. aegypti* is thought to have African origins (Holmes and Twiddy, 2003; Wilder-Smith and Gubler, 2008). On the other hand, the fact that all four serotypes of the dengue virus appear in humans and primates in Asia strongly suggests that the cross-species transmission of the four serotypes occurred independently of each other. (Holmes and Twiddy, 2003; Wilder-Smith and Gubler, 2008).

Several recent findings suggest that the origin of certain dengue virus serotypes, especially specific genotypes, is somewhere in Southeast Asia. A study performed by Villabona-Arenas and Zanotto in 2011 concluded after studying 310 DENV-4 samples from 37 countries between 1956 and 2008 that the most probable origin of DENV-4 was Malaysia or Thailand. More specifically, genotypes I and II subclade II coincided with the ancestral

occurrences of dengue in Malaysian monkeys. To further support Villabona-Arenas and Zanotto's observations other studies have contributed molecular evidence that also suggests the origin of DENV-4 is Southeast Asia, most likely Thailand or Malaysia (Klungthong et al., 2004). When observing a different serotype, DENV-3, researchers found that the strain first appeared in 1890 with the major branching of genotypes occurring approximately 30 to 40 years ago in Thailand, Indonesia, and Sri Lanka (Araújo et al., 2009).

With respect to DENV-1, Patil et al. investigated different Indian genotype isolates sampled between 1962 and 2005. They discovered that these isolates were introduced into India through African and Singaporean roots and then exported to the Americas around the middle of the 20th century. Another noteworthy observation from their study was that the DENV-1 is approximately 123 years old (which is consistent with previous findings) and that the American-African genotype shared a common node with the Malaysian sylvatic genotype around that predicted time period. Lastly, a study conducted in 2004 by Foster et al. presented information that indicated that the 59 DENV-2 isolates collected between 1981 and 2000 from ten of the Caribbean islands, two Central American countries, and six South American countries were of Southeast Asian origin. From these different phylogenic and evolutionary studies on all four serotypes of the dengue virus, scientists can confidently infer that the virus has some form of Asian origin. However, in order to actually pinpoint a particular region, further investigations into the geographical migration and the molecular time-scale of the dengue virus is needed.

Immunological Response. The dengue virus displays antibody dependent enhancement, which presumably leads to DHF and DSS (Guzmán and Kourí, 2004; Kliks et al., 1989; Sadon et al., 2008; Schmidt, 2010). Antibody-dependent enhancement (ADE) is a

disease spreading mechanism that causes individuals with a secondary infection to be more infectious, as compared to when they had a primary infection by a different strain (Billings et al., 2008). In most immunological processes, the human body produces antibodies in response to an infection in order to provide protection against a particular pathogen as well as similar pathogens (Adams and Boots, 2006). However, in some instances, such as the four strain dengue virus, ADE occurs and the antibodies developed in response to the first serotype infection form a complex with the second serotype. This actually facilitates the entry of the virus into more cells, thus increasing viral production (Adams and Boots, 2006; Billings et al., 2008).

Recent research has revealed that cross-reactive non-neutralizing antibodies acquired after the primary infection have the potential to mediate an ADE of the second heterologous infection with the use of Fc receptors located on monocytes or macrophages (Guzmán and Kourí, 2004; Kliks et al., 1989; Schmidt, 2010; Sun et al., 2011). The three classes of FcγR serve as receptors on monocytes or macrophages for dengue virus entry in the forms of virus-antibody immune complexes (Sun et al., 2011). Studies have shown that the highest binding affinity to these receptors corresponds with infection enhancement as well as suboptimal concentrations of the primary infection antibodies (Sun et al., 2011).

From this discovery, researchers inferred that once the antibodies for the primary infection have dropped below a certain threshold, those antibodies fail to neutralize other serotypes of the dengue virus and instead help them enter the cells bearing the dengue receptors (Kliks et al., 1989; Schmidt, 2010; Sun et al., 2011). An additional correlation detected that IL-10, TNF-α, and IFN-α, cytokines produced by monocytes or macrophages, were much more elevated in patients with secondary infections than those with primary

infections. By over secreting cytokines, there is a dangerous increase in the inflammatory response and cell permeability which is likely the cause of vesicular leakage and thrombocytopenia in DHF and DSS patients (Schmidt, 2010; Sun et al., 2011).

Scientists have also discovered that CD4+ and CD+8 T cells play an important role in the ADE process (Guzmán and Kourí, 2004). When infected with a second serotype, memory T cells target the dengue virus-infected monocytes. The cells are lysed by cytokines. The release of enzymes and activators from within the cell causes plasma leakage and can potentially cause shock (Guzmán and Kourí, 2004). A research article published by Friberg et al. (2011) was the first to assess the cross-reactivity of the CD8+ repertoire produced after a primary infection in HLA-A* 1101+ individuals using *in vitro* binding with homologous and heterologous peptide variants found in the four DENV serotypes. The reasoning behind demonstrating cross-reactivity using HLA-A* 1101+ individuals is that researchers have discovered that it is the common haplotype circulating within DENV endemic areas and has an association with the susceptibility of the dengue disease. This study demonstrated that sequential infections of DENV-1 and DENV-3 activate cross-reactive T cells that vigorously produce IFNγ. They also found that a secondary infection by DENV-2 caused poor T cell activity, but actually triggered an increase in TNFα production by monocytes that contribute to an increase in disease severity. Their results provided critical evidence that supports the previous epidemiological findings, which indicated that the order of the different DENV infections influences the effects of antibody dependent enhancement and the final clinical outcome a person experiences.

From these studies, scientists have made strides into understanding this unique phenomenon and how it molecularly influences the dangerous symptoms experienced with

DHF and DSS. The collective findings of these and future studies can be utilized to develop vaccines, diagnose patients, and increase the effectiveness of treating persons infected with dengue.

Vaccine Development. Dengue infections continue to increase in countries already affected by the virus, and now, as current epidemiological evidence shows, the virus is spreading to new areas such as the Middle East and the United States. Due to these findings, the development of an effective vaccine has become a main priority for many infectious disease researchers (Chao et al., 2012; Shepard et al., 2004). There is no doubt that even with diligent vector control, a vaccine is the only solution to decrease the number of infections that occur worldwide.

ADE has turned the vaccination process for the dengue virus into quite the on-going challenge (Billings et al. 2008). Separate vaccinations in a series have been considered, but because of ADE, they pose a threat and are not believed to be the preventive method (Billings et al. 2008). Single serotype vaccines have been considered; however, if patients received a vaccine against a single serotype, they would only be protected against that particular serotype. Whitehead and colleagues constructed a DEN-4 vaccine candidate that produced 100% neutralizing antibody seroconversion in 24 adult volunteers (Billings et al. 2008). This vaccine candidate could be applicable to an area of the world that only has that one serotype in existence, but regions of the world where dengue is a threat typically contain two or more serotypes. Thus, vaccination could actually do more harm than good, since an infection by a second serotype would be enhanced and cause increased health risks (Billings et al. 2008).

An optimal vaccine would need to protect against all strains of the disease simultaneously in order to prevent the increased transmission of the strains not covered (Billings et al. 2008). To further complicate development, such a vaccine could be particularly dangerous to individuals who already have antibodies for a particular serotype of dengue. Furthermore, previously infected persons and infants born to a dengue-immune mother are at risk (Billings et al. 2008; Kliks et al. 1989). Despite this, a tetravalent vaccine is seen as the most viable course of action (Bakshi, 2007; Thomas, 2011). A number of research facilities and companies are currently working on developing a tetravalent DENV vaccine and they are showing promising results.

An article published by Watanaveeradej et al. tested the safety and immunogenicity of a tetravalent live-attenuated dengue vaccine in healthy infants who had not yet been infected with any of the four dengue serotypes (2011). A vaccine candidate was developed by the Walter Reed Army Institute of Research (WRAIR) and GlaxoSmithKline Biologicals (GSK) in hopes of protecting children and adults against all forms of the dengue virus. The vaccine candidate was transported to Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok to be tested on Thai infants between 12 and 15 months of age. When preparing the vaccines, monovalent vaccines for each specific DENV serotype were developed and then blended together to make a tetravalent vaccine prior to injection. Each subject received two doses of the vaccine candidate in either full-dose or low-dose (1/10) forms spread over a six month period and had their neutralizing antibodies measured. The researchers also noted any reactions experienced by the recipients.

The vaccine candidates showed promising results. Both the full-dose and low-dose vaccines were well-tolerated by recipients with only minor cases of redness, swelling, and

pain at the injection site and fever for no longer than 3 days after the administration of each dose. This was similar to side effects shown with the control vaccine (Varicella and H. influenzae type B) recipients. None of the recipients experienced hemorrhaging or other serious adverse events from the administration of the DENV vaccines. The investigators determined that the low-dose vaccine was no clinically safer than the full-dose vaccine. With respect to immunogenicity, researchers were unable to detect any neutralizing antibodies in the control group but were able to detect them in the low-dose and full-dose recipients. The full-dose vaccine presented the best results with 85.7% of its recipients displaying trivalent or tetravalent neutralizing antibodies 30 days after the second dose. The low-dose vaccine presented lower immunogenicity and, therefore, would not be the best method of vaccination. Unfortunately, there was a significant decrease in neutralizing antibodies in the full-dose vaccine recipients a year after the second dose was administered especially with DENV-1 and DENV-3. This suggests that something occurs within the first year of immunization and must be studied in order to produce an effective vaccine with long-term protection.

This study presents great promise with respect to vaccine development for Thailand and the other countries currently at risk for dengue infections. Because the full-dose DENV vaccine was just as safe as the low-dose DENV but provoked a better immunological response particularly with DENV-2 and DENV-4, it can be concluded that a full-dose DENV vaccine is probably the best method of vaccination. They also determined that the age group used in this trial is an appropriate age to distribute a DENV vaccine. Nonetheless, this study introduces important questions that need to be answered to increase the immunogenicity of future DENV vaccine candidates (Watanaveeradej et al., 2011).

The vaccine candidate presented in the article above is one of the many vaccine candidates presently being tested in clinical trials (Chao et al., 2012). According to the WHO, the DENV vaccine candidate that has shown the most promising results, and is currently the farthest along in the clinical development process, is a live-attenuated tetravalent vaccine produced by Sanofi Pasteur. It is actually a chimeric yellow fever-dengue fever vaccine that is being evaluated in a phase IIb study which is taking place in the Ratchaburi Province, Thailand with 4,002 children between 4 and 11 years of age. In addition to this, a phase III study is underway in 31,000 children and adolescents in 10 countries in Asia and Latin America (WHO, 2012).

Research indicates that a tetravalent vaccine is the best possible solution and will be the only means of decreasing dengue infection rates (Chao et al., 2012). Due to the complications discussed and presented by Chao et al., as well as various others, development of an effective vaccine to prevent the spreading of the dengue virus will be an on-going challenge. Until these challenges are overcome, there is currently no safe, licensed dengue vaccine available to the public and it is predicted that there will not be one for another 2 to 5 years (Chao et al., 2012).

Research Project. After evaluating the impact of the dengue, it is easy to see that scientific progress needs to be made to further understand the virus. In order to do so, detection of all four serotypes is the essential first step. Due to the fact that the dengue virus is one of the biggest threats to humanity in the country of Thailand, AFRIMS in Bangkok has become an important research facility in the country and one of the leading global research facilities for studying the dengue virus, as well as other major health threats to humanity such

as HIV, influenza, and Japanese encephalitis. AFRIMS was the site where the laboratory research portion of this thesis was conducted.

Since 1994, AFRIMS has used a modified Lanciotti's conventional reverse transcriptase polymerase chain reaction (RT-PCR) method to diagnose the dengue virus; however, the analysis of PCR results of 13,532 previously confirmed dengue cases by enzyme-linked immunosorbent assay (ELISA) tested over 11 years (2000-2010) showed 17-42% negative conventional RT-PCR results (AFRIMS's data). These results sparked a new research objective: to improve the detection and identification of the DENV in these dengue ELISA confirmed samples that were missed by conventional RT-PCR results. Because earlier studies analyzed only the positive RT-PCR samples, this increased the probability of missing possible dengue virus mutants. This study was one of the first to actually analyze previously negative RT-PCR samples to determine the efficiency of each method, and thus improve the system.

Once the new research project was approved, laboratory personnel began making the effort to retrieve and analyze data to improve the previous method of detecting the dengue virus from specimens in the areas surrounding Bangkok. The prior methods included QRT-PCR paired with either virus isolation in C6/36 cells with typing ELISA or mosquito inoculation followed by dengue ELISA. These two approaches were used to verify that the conventional RT-PCR cannot detect some DENV variances. The positive samples by one of these two methods were examined for the conventional RT-PCR's primer binding site regions on the DENV genome. New primers were designed based off the DENV mutants. Conventional RT-PCR tests were performed using these new primers to improve the sensitivity of the assay.

MATERIALS AND METHODS

Purpose. It was hypothesized that by discovering mutant strains of the dengue virus currently circulating in the Bangkok, Thailand region by assessing the negative conventional RT-PCR which tested positive with ELISA, new primers could be designed to improve the detection of arising mutant strains. By continuously improving methods of dengue virus detection, accurate research can be conducted which will provide advantageous information into the production of an effective vaccine and thus improve protection of mankind.

Samples. 300 serum specimens, which previously tested positive for dengue primary and secondary infection using the AFRIMS Dengue IgM/IgG ELISA and negative by conventional RT-PCR, were randomly selected to be tested by in-house TaqMan^{TM} real time RT-PCR. Among these selected 300 samples, 35 were selected for virus isolation with ELISA typing. The samples with positive TaqMan^{TM} real-time RT-PCR and/or virus isolation with typing ELISA were then examined for the conventional RT-PCR's primer binding site by sequencing.

Viral RNA Extraction and Modified Lanciotti's conventional RT-PCR. Viral RNA was extracted from serum specimens using QIAgen viral RNA extraction kits and following the manufacturer's instructions. Modified Lanciotti's conventional RT-PCR was performed in accordance with the method described in the Samples section above (Klungthong et al, 2007).

Quantitative real time RT-PCR. The quantitative RT-PCR was performed by following AFRIMS's standard operating procedure and provided for use in this study (Sadon et al., 2008).

Virus isolation in C6/36 cells with typing ELISA. Virus isolation in C6/36 was performed with 35 samples in accordance with the previously described method (Klungthong et al, 2007). The positive isolates were then tested by typing ELISA. First, the microtiter wells were coated with goat anti-mouse IgG and left at 4°C for approximately 20 hours and then a specific monoclonal antibody was added to the wells. Following the addition of the first antibody which remained at room temperature for two hours, the test specimen was inserted into the well. After sitting at 4°C for approximately 20 hours, the wells were washed and the human anti-flavivirus IgG- HRP (second antibody) was added. Lastly, after sitting at 37°C for one hour, the wells were washed a final time and the OPD substrate was added and the reaction was stopped. Positive results were then identified by the appearance of a yellow-orange color inside the well.

New primer design for conventional RT-PCR. All positive dengue isolates were subjected for sequencing of the primer binding sites on DENV genome to examine mutation. All obtained sequences were aligned with Lanciotti's primers using Sequencher software. The new primers were designed to avoid the mismatch points between the primers and templates, then tested for efficiency on seven specimen that were known for containing dengue mutants.

Conventional RT-PCR with new primers and gel electrophoresis. Due to the efficiency of the new primers, we decided to test them using conventional RT-PCR and gel electrophoresis analysis on 50 RNA samples that had been stored at -70°C for approximately 4 months.

Comparison of test results to clinical analysis. In order to determine if trends existed between the results from qRT-PCR, ELISA, and conventional RT-PCR with the new primers compared to the clinical analysis received from Queen Sirikit National Institute of Child Health and the hospital in Kamphaeng Phet, detection of the dengue virus positive results were compared with the disease severity (DF vs. DHF), type of infection (primary vs. secondary), temperature ($\geq$ 38°C vs. < 38°C) and number of days with fever (0-4 vs. >4). In addition, this method of the research was used to address variation in the sensitivity between the different tests.

RESULTS

Quantitative real time RT-PCR. Quantitative RT-PCR was performed on 300 samples of dengue ELISA confirmed cases with negative conventional RT-PCR of which 180 (60%) samples appeared positive for the dengue virus (Fig. 1). From those 180 samples that tested positive for the dengue virus, 129 (71.7%) were DEN-1, 39 (21.7%) were DEN-2, 4 (2.2%) were DEN-3, and 8 (4.4%) were both DEN-1 and DEN-2.

Virus isolation in C6/36 cells with typing ELISA. Virus isolation in C6/36 cells with typing ELISA performed with 35 samples selected from the 300 samples was able to identify 8 (22.86%) positive dengue isolates (Fig. 2). Of those 8 positive results, 4 (50%) were DEN-1, 3 (37.5%) were DEN-2, and 1 (12.5%) was DEN-3.

New primer design conventional RT-PCR (Fig.3). Sequences of the primer binding sites on the genome of all positive DENV isolates revealed two mismatches in the D1 primer (forward primer), nine mismatches in the D2 (universal reverse primer), five mismatches in the TS1 (DEN-1 specific reverse primer), two mismatches in TS2 (DEN-2 specific reverse primer), and two mismatches in TS3 (DEN-3 specific reverse primer). From these mismatches caused by the dengue virus mutants, new primers were designed with the hope of improving the detection of the dengue virus by conventional RT-PCR. These primers were then tested on the seven specimens known for containing the dengue mutants and displayed 100% efficiency.

Conventional RT-PCR with new primers and gel electrophoresis. After running the 50 samples through conventional RT-PCR with the new primers and gel electrophoresis,

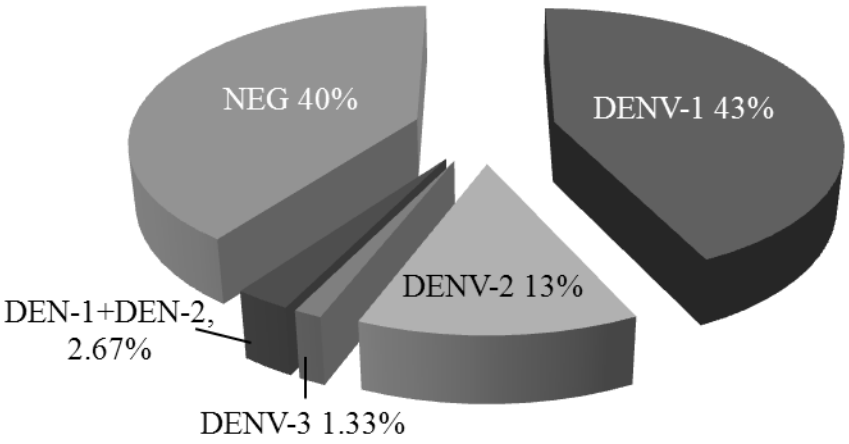

Figure 1. Percentage of qRT-PCR positive and negative of 300 DEN confirmed cases with negative conventional RT-PCR results. *Performed and provided by laboratory personnel.

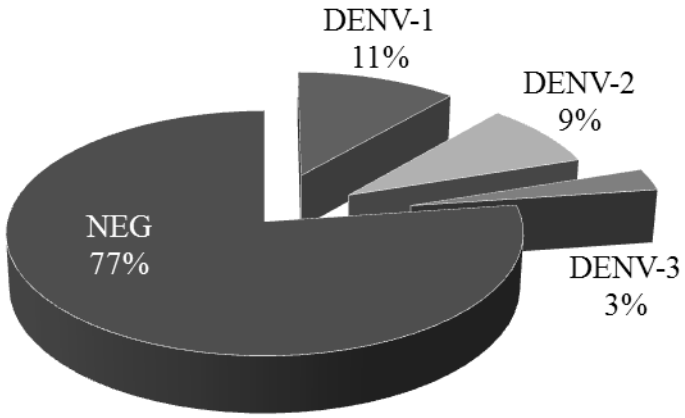

Figure 2. Percentage of ELISA DENV positive and negative of the 35 samples that originally appeared negative by conventional RT-PCR.

Forward Primer D1 (based on both DEN-2 and 3 mutants):

5’-TCAATA TGCTGAAACG CG(A,T)GAGAAAC CG-3’

Reverse Primer D2 (based on DEN-1, 2 and 3 mutants):

5’- TTGCACCAACAGTCTATGTCTTCTGGCTC-3’

5’ TTGCACCAACAGTCAATGTC(T,A)TC(A,T)GG(T,C)TC-3’

Reverse Primer TS1 (based on DENV-1 mutants):

Reverse: 5’- CGCCTCAGTGATTC(T,G)AGG-3’

Reverse Primer TS2 (based on DENV-2 mutants):

Reverse: 5’- AGCCACCAGGGCCATGAACAC-3’

Reverse Primer TS3 (based on DENV-3 mutants):

Reverse: 5’- GGTAACATCATCAT(A,G)AGACAGA-3’

Figure 3. New primer sequences based off dengue mutants with mismatch sites shown in parentheses. For primers with mismatches shown, more than one primer was synthesized to incorporate multiple mutations at mismatch sites.

11 (22%) were positive for the dengue virus (Table 1). Of those 11 positive samples, six (54.5%) were DEN-1, four (36.4%) were DEN-2, and one (9.1%) was DEN-3. When comparing the results of these positive samples with their qRT-PCR results, ten conventional RT-PCR samples matched their qRT-PCR serotype and one sample was positive for DEN-1 by conventional RT-PCR but negative by qRT-PCR.

Comparison of test results to clinical analysis (Table 2). For persons classified as having DF, 65.9% of positive samples were detected by qRT-PCR, 27.8% of the positive samples were detected by ELISA, and 20.8% of the positive samples were detected by conventional RT-PCR. For those having DHF, 39.3% of positive samples were detected by qRT-PCR, 20% of the positive samples were detected by ELISA, and 22.4% of positive samples were detected by conventional RT-PCR.

For persons classified as having a primary infection, 76.3% of positive samples were detected by qRT-PCR, 50% of positive samples were detected by ELISA, and 42.9% of positive samples were detected by conventional RT-PCR. For those having a secondary infection, 55.7% of positive samples were detected by qRT-PCR, 19.4% of positive samples were detected by ELISA, and 14.3% of positive samples were detected by conventional RT-PCR.

For persons classified as having a fever of 38°C or higher, 61.7% of positive samples were detected by qRT-PCR, 38.5% of positive samples were detected by ELISA, and 33.3% of positive samples were detected by conventional RT-PCR. For those having a fever less than 38°C, 60.1% of positive samples were detected by qRT-PCR, 15% of positive samples

qRT-PCR result	# of Samples	Nested-PCR results w/ New Primer	# of Samples
DEN-1	20	DEN-1	5
		NEG	15
DEN-2	18	DEN-2	4
		NEG	14
DEN-3	2	DEN-3	1
		NEG	1
NEG	10	DENV-1	1
		NEG	9

Table 1. Conventional RT-PCR (new primers) results with the 50 RNA samples (kept in 70°C for 4 months) previously tested by qRT-PCR.

Analysis	qRT-PCR	Virus Isolation	Conventional-PCR (New Primer)
DF	65.79	27.78	20.83
DHF	39.34	20	22.37
Primary	76.27	50	42.86
Secondary	55.65	19.35	14.29
Fever Day 0-4	63.3	15.63	30
Fever Day >4	57.4	100	16.67
Temp. >=38°C	61.73	38.46	33.33
Temp. <38°C	60.1	15	18.42
Serotype	DEN-1,2,3	DEN-1,2,3	DEN-1,2,3

Table 2. Percentage of positive results in comparison to clinical analysis.

were detected by ELISA, and 18.4% of positive samples were detected by conventional RT-PCR.

Lastly, for patients classified as having a fever for up to 4 days, 63.3% of positive samples were detected by qRT-PCR, ELISA detected 15.6% of the positive samples, and 30% of positive samples were detected by conventional RT-PCR. For those patients classified as having a fever for more than four days, 57.4% of positive samples were detected by qRT-PCR, ELISA detected 100% of the positive samples, and 16.7% of positive samples were detected by conventional RT-PCR. It must also be noted that only DENV-1, 2, and 3 were detected since those were the strains with detectable mutants while DENV-4 was not.

DISCUSSION

Analysis of Study. Once the results were compared and analyzed it was apparent that each method can detect the dengue virus differently depending on the patient's clinical analysis. When analyzing the results for disease severity, qRT-PCR (65.8%) and ELISA (27.78%) detected the dengue virus better in samples from DF patients than DHF patients. Conventional RT-PCR detected the dengue virus fairly equally in samples from DHF patients and DF patients. These results suggest that when testing patient serum in those suspected of DF, qRT-PCR and ELISA are more reliable. On the other hand, when testing patient serum in those suspected of DHF, conventional RT-PCR or qRT-PCR should be used.

When analyzing the results for infection type, all three of the methods detected the dengue virus in samples from patients with a primary infection better than in samples from patients with a secondary infection suggesting that all these tests are better at detecting the dengue virus in a primary infection. It is important to note that qRT-PCR showed a higher percentage (76.3% for primary and 55.7% for secondary) of detection for both types of infection overall. Because of this finding, it is suggested that qRT-PCR be used to test serum samples in both primary and secondary infections rather than ELISA and conventional RT-PCR.

When analyzing the results for degree of temperature, ELISA (38.5%) and conventional RT-PCR (33.3%) detected the dengue virus in samples from patients with a fever of 38°C or higher better than in samples from patients with a fever less than 38°C, while qRT-PCR detected both fever temperature's samples fairly equally. Since qRT-PCR

was able to detect the virus with the highest percentage (~61.0%) at both temperature ranges, it is suggested that qRT-PCR be used with regards to these conditions.

Lastly, when analyzing the results based on number of days with fever, qRT-PCR (63.3%) and conventional RT-PCR (30.0%) detected the dengue virus in samples from patients who had a fever up to 4 days better than in samples from patients who had a fever greater than 4 days. ELISA (100%), however, was able to detect the dengue virus better in samples from patients who had a fever for more than four days than in samples from patients who had a fever for 0-4 days. From these results, qRT-PCR and conventional RT-PCR should be used to detect the virus in sera from patients with a fever for up to 4 days while ELISA should be used for those with fevers greater than 4 days.

It can be concluded from the results that qRT-PCR typically was the best of the three tests when detecting the dengue virus. These findings are consistent with those in a previous study where researchers assessed the effectiveness of dengue virus detection when using qRT-PCR and conventional RT-PCR (Sadon et al., 2008). These researchers found that both qRT-PCR and conventional RT-PCR provide similar sensitivity but one can provide more reliable results depending upon the serotype. QRT-PCR was found to be more sensitive for DENV-1 and DENV-3 while conventional RT-PCR was found to be more sensitive for DENV-4 (Sadon et al., 2008). ELISA was suggested to be less efficient and accurate due to cross-reactivity among flaviviruses and the fact that it requires more time (Sadon et al., 2008). This information should be received with caution, however, because according to other findings conducted across various fields of microbiology, qRT-PCR can be less sensitive than conventional RT-PCR and can also lose sensitivity if performed too fast (Bastian et al., 2008; Hilscher et al., 2005). Because of the variations among detection rates

as compared to the clinical analyses, each method is important in the detection and therefore to the research being conducted on the dengue virus. Our study was one of the first to actually analyze previously negative RT-PCR samples to determine the efficiency of each method. In contrast, earlier studies analyzed only the positive RT-PCR samples, likely increasing the potential of missing possible dengue virus mutants.

Caution must be taken when collecting specimens because the dengue virus is easily altered by heat and must be delivered to the laboratory fairly quickly in order to isolate the virus, or it must be stored for a short period of time at 4°C (Guzmán and Kourí, 2004). In this research, the RNA samples were stored for four months at -70°C as recommended but after reviewing the results, it is suggested that fresh RNA samples be utilized for the future. Though proper storage methods can increase the life of RNA samples, there is a possible risk of RNAse contamination and RNA degradation due to alkaline hydrolysis by the additional 2' hydroxyl group present in RNA (IDT, 2013). Such fresh samples should be used in conjunction with updated PCR primers and compared to the clinical analysis acquired so that AFRIMS can continue to improve the detection and diagnosis of the dengue virus in the circulating area of Bangkok, Thailand. In the future, the sample sizes used in assessing conventional RT-PCR and ELISA should be increased in order to decrease the chances of sampling error.

For additional improvement, researchers will implement external quality assurance (EQA) which assesses the efficiency and accuracy of dengue molecular diagnosis methods applied by expert laboratories (Domingo et al., 2010). Finally, this research study provided the laboratory personnel at AFRIMS with a further understanding of how the dengue virus is mutating in Bangkok, Thailand and the surrounding areas. Since serology is considered to be

the widely used method of confirming the diagnosis of dengue in a patient through methods such as those in this study, the findings from this research are important for future diagnosis and possible vaccination production (Guzmán and Kourí, 2004; Nisalak et al., 2003; Jarman et al., 2011).

Conclusion. Ever since the first major outbreak of DHF in Bangkok in 1958, the dengue virus has been the research focus for Thailand as well as much of the world. It continues today to be one of the top infectious diseases posing a global threat. Preventive measures have been enforced to keep the virus from spreading but the only way to defeat the virus and bring infection rates to a lower level is through vaccine development and distribution. There are currently several tetravalent vaccine candidates advancing to clinical trials; however, their development must conquer the many challenges the dengue virus presents, which will involve a few more years of observation and research. In order to obtain such a scientific feat, research conducted on a molecular scale is essential and must be performed with the upmost efficiency. The research presented in this thesis provides information for the improvement of detecting and diagnosing the dengue virus so that scientists can track it and better understand how it is evolving. Furthermore, this research indirectly affects the advances made in vaccine development which in turn helps in the fight against the dengue virus and is critical for the improvement of global health.

LITERATURE CITED

Adams B, Boots M. Modelling the relationship between antibody-dependent enhancement and immunological distance with application to dengue. J Theoretical Bio 2006; 242:337-346.

Araújo JMG, Nogueira RMR, Schatzmayr HG, Zanotto PMA, Bello G. Phylogeography and evolutionary history of dengue virus type 3. Infect Gen Evo 2009; 9: 716-725.

Bakshi AS. Dengue fever, DHF and DSS. Apollo Med 2007; 4: 111-117.

Barbazan P, Yoksan S, Gonzalez JP. Dengue hemorrhagic fever epidemiology in Thailand: description and forecasting of epidemics. Micro Infect 2002; 4: 699-705.

Bastien P, Procop GW, Reischl U. Quantitative Real-Time PCR is not more sensitive than conventional PCR. J Clin Microbiol 2008; 46: 1897-1900.

Billings L, Fiorillo A, Schwartz IB. Vaccinations in disease models with antibody-dependent enhancement. Math Biosci 2008; 211:265-281.

CDC, Dengue, Center for Disease Prevention and Control, Atlanta, 2013. http://www.cdc.gov/dengue/.

Chao DL, Halstead SB, Halloran ME, Longini IM Jr. Controlling dengue with vaccines in Thailand. Plos Negl Trop Dis 2012; 6: e1876 1-11.

Domingo C, Niedrig M, Teichmann A, Kaiser M, Rumer L, Jarman RG, et al. 2nd international external quality control assessment for the molecular diagnosis of dengue infections. Plos Neg Trop Dis 2010; 4 (10): e833 1-9.

Foster JE, Bennett SN, Carrington CVF, Vaughan H, McMillan WO. Phylogeography and molecular evolution of dengue 2 in the Caribbean basin, 1981–2000. Viro 2004; 324: 48-59.

Friberg H et al. Memory CD8+ T cells from naturally-acquired primary dengue virus infection are highly cross-reactive. Immunol Cell Bio 2011; 89: 122-129.

Gibbons RV. Dengue Conundrums. Inter J Antimicrob Agents 2010; 36S: 36S-39S.

Gubler DJ. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 2002; 10: 100-103.

Guzmán MG, Kourí G. Dengue diagnosis, advances and challenges. Inter J Inf Dis 2004; 8: 69-80.

Hilscher C, Vahrson W, Dittmer DP. Faster quantitative real-time PCR protocols may lose sensitivity and show increased variability. Nucleic Acids Res 2005; 33: e182 (1-8).

Holmes EC and Twiddy SS. The origin, emergence and evolution genetics of the dengue virus. Infect Gen Evo 2003; 3: 19-28.

IDT. Unraveling RNA – The importance of a 2' hydroxyl. Integrated DNA Technologies 2013. http://www.idtdna.com/pages/decoded/decoded-articles/core-concepts/decoded/2011/03/16/unraveling-rna-the-importance-of-a-2'-hydroxyl.

Jansen CC, Beebe NW. The dengue vector Aedes aegypti: what comes next. Microbes Infect 2010; 12: 272-279.

Jarman RG, Nisalak A, Anderson KB, Klungthong C, Thaisomboonsuk B, Kaneechit W, et al. Factors influencing dengue virus isolation by C6/36 cell culture and mosquito inoculation of nested PCR-positive clinical samples. Am J Trop Med Hyg 2011; 84 (2): 218-23.

Kliks SC, Nisalak A, Brandt WE, Wahl L, Burke D. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am J Trop Med Hyg 1989; 40: 444-451.

Klungthong C, Gibbons RV, Thaisomboonsuk B, Nisalak A, Kalayanarooj S, Thirawuth V, et al. Dengue virus detection using whole blood for RT-PCR and viral isolation. J Clin Microbiol 2007; 45: 2480-2485.

Klungthong C, Zhanga C, Mammen MP Jr., Ubolb S, Holmes EC. The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. Viro 2004; 329: 168-179.

Nisalak A, Endy TP, Nimmannitya S, Kalayanarooj S, Thisayakorn U, Scott RM, et al. Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am J Trop Med Hyg 2003; 68 (2): 191-202.

Parker D, Holman D. Event history analysis of dengue fever epidemic and inter-epidemic spells in Barbados, Brazil, and Thailand. Inter J Infect Dis 2012; 16: e793-798.

Patil JA, Cherian S, Walimbe AM, Patil BR, Sathe PS, Shah PS, Cecilia D. Evolutionary dynamics of the American African genotype of dengue type 1 virus in India (1962–2005). Infect Gen Evo 2011; 11: 1443-1448.

Poersch C, Pavoni D, Queiroz M, Borba L, Goldenberg S, Santos C, Krieger M. Dengue virus infections: comparison of methods for diagnosing the acute disease. J Clin Viro 2005; 32: 272-277.

Sadon N, Delers A, Jarman RG, Klungthong C, Nisalak A, Gibbons RV, Vassilev V. A new quantitative RT-PCR method for sensitive detection of dengue virus in serum samples. J Viro Meth 2008; 153: 1-6.

Schmidt AC. Response to dengue fever – the good, the bad, and the ugly. New Engl J Med 2010; 363:484-487.

Shepard DS et al. Cost-effectiveness of a pediatric dengue vaccine. Vaccine 2004; 22: 1275-1280.

Srikiatkachorn A, Gibbons RV, Green S, Libraty DH, Thomas SJ, Endy TP, et al. Dengue hemorrhagic Fever: The sensitivity and specificity of the World Health Organization Definition for Identification of Severe Cases of Dengue in Thailand, 1994-2005. Clin Infect Dis 2010; 50: 1135-43.

Sun, P. et al. Infection and activation of human peripheral blood monocytes by dengue viruses through the mechanism of antibody-dependent enhancement. Viro 2011; 421:245-252.

Thomas SJ. The necessity and quandaries of dengue vaccine development. J Infect Dis 2011; 203: 299-303.

Tropical Medical Bureau. Major increase in Dengue cases throughout Thailand during 2012. Tropical Medical Bureau, Ireland. 2013. http://www.tmb.ie/destinations/news.asp?id=188563.

Villabona-Arenas CJ, Zanotto PMA. Evolutionary history of dengue virus type 4: Insights into genotype phylodynamics. Infect Gen Evo 2011; 11: 878-885.

Watanaveeradej V et al. Safety and immunogenicity of a tetravalent live-attenuated dengue vaccine in flavivirus-naive infants. Am J Trop Med Hyg 2011; 85: 341-351.

Weaver SC, Vasilakis N. Molecular evolution of dengue viruses: Contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect Gen Evo 2009; 9: 523-540.

WHO. Dengue guidelines for diagnosis, treatment, prevention and control. World Health Organization 2009. http://whqlibdoc.who.int/publications/2009/9789241547871_eng.pdf.

WHO. Questions and answers on dengue vaccines: phase IIb study of CYD-TDV. World Health Organization 2012. http://www.who.int/vaccine_research/committees/WHO_dengue_vaccine_QA_september2012.pdf.

WHO, Dengue and Severe Dengue, World Health Organization, Geneva, 2013. http://www.who.int/mediacentre/factsheets/fs117/en/.

Wichmann O et al. Dengue in Thailand and Cambodia: An assessment of the degree of underrecognized disease burden based on reported cases. Plos Negl Trop Dis 2011; 5: e996 (1-9).

Wilder-Smith A, Gubler D. Geographic expansion of dengue: The impact of international travel. Med Ethics North Am 2008; 22: 1275-1280.